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Optogenetic Activation of the Sublaterodorsal (SLD) Nucleus Induces Rapid Muscle Inhibition

prepared by Cameron H Good

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70 President St, Charleston, South Carolina

under contract 1120-1120-99

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The sublaterodorsal (SLD) nucleus is hypothesized to induce muscle atonia during rapid eye movement (REM) sleep. This was tested by injecting channelrhodopsin into the SLD of 5 rats, followed by a series of behavioral tests. Results confirmed our ability to rapidly induce muscle atonia following optogenetic stimulation of the SLD, suggesting that activation of the SLD is sufficient to cause a REM atonia like state.					
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1. Introduction

Sleep occurs in virtually all animals, and is essential for normal brain function, but its underlying mechanisms and functions are surprisingly poorly understood. For example, the ultimate purpose of sleep, and the mechanisms driving it, are almost completely unknown despite intensive efforts over many decades. The transition between waking and sleep, and non-rapid eye movement to rapid eye movement (NREM/REM) sleep, involves rapid state changes that are physiologically distinct in their impact on sensory perception, muscle tone and neural activity, as measured with electroencephalography (EEG). One of the hallmarks of rapid eye movement (REM) sleep is muscle inhibition and is generally referred to as muscle atonia to describe the loss of muscle tone. REM sleep atonia is a consequence of gamma-aminobutyric acid (GABA) and glycine inhibition of spinal motor neurons that results in a paralysis like state of nearly all peripheral muscles (except respiratory and oculomotor).^{1,2} The neural circuitry that drives atonia is thought to originate in the pontine reticular activating system, while injections of cholinergic agonists (i.e., carbachol and neostigmine) in the brainstem of awake cats and rats can rapidly induce a REM sleep-like state complete with muscle atonia.^{3–10} The most efficacious location for these injections is a region termed the dorsal part of the pontis oralis (PnO) and pontis caudalis (PnC), also named the peri-locus coeruleus α (peri-LCo) in cat, and the equivalent structures in rodents named the sublaterodorsal (SLD) nucleus or dorsal subcoeruleus (SubCD). Disinhibition of the SLD with injections of bicuculline or gabazine (GABA_A antagonists) induces a REM-like state characterized by muscle atonia, EEG activation and nonreactivity to surrounding stimuli,¹¹ while similar results are found when the peri-LCo is activated by the specific glutamate agonist kainate.^{11,12} Lesioning the ventral SLD with ibotenic acid caused loss of atonia in almost every REM sleep episode,^{13,14} analogous to humans with localized brainstem infarcts of this region.¹⁵ Together, these results confirm the SLD as necessary for REM sleep atonia, thus making it an ideal target for exploration.

Interestingly, SLD regulation of the different components of REM sleep may be neuroanatomically dissociable. Ventrally located SLD (vSLD) glutamate neurons drive atonia via projections to the gigantocellular nucleus (Gi), while dorsal SLD cells regulate cortical EEG theta rhythms through relay nuclei, including projections to the medial septum (MS).^{14,16} Thus, it may be possible to activate the vSLD atonia circuitry without impacting cognition. Alternatively, it could be possible to activate the entire structure to turn on both the cognitive and motor components of REM sleep. To date no one has attempted to optogenetically manipulate the SLD, making it a prime experimental target for exploring rapid

changes in cognition and atonia. Such optogenetic approaches would show direct causal linkages on a much faster time scale than the prior studies previously cited that relied on pharmaceuticals to induce REM sleep. Building on this known literature, we have generated pilot data showing that we can rapidly induce atonia by optogenetically stimulating the SLD region (see Section 3 for details). Interestingly, we were able to produce this effect without an apparent increase in cortical EEG theta; this is likely to be the first demonstration of such a clear dissociation of these 2 components of REM sleep.

2. Methods

Animals: Six adult male Sprague-Dawley rats (275–350 g, Charles River Laboratories, Raleigh, North Carolina) were used for these experiments. All Protocols were conducted under National Institutes of Health (NIH) Guidelines using the NIH handbook “Animals in Research” and were approved by the Animal Care and Use Committee at the Medical University of South Carolina (MUSC).

Surgery: Rats were initially anesthetized with 5% isoflurane/95% oxygen. During this time their heads were shaved and placed in stereotactic ear bars. Once the animals were secured in place, the isoflurane was gradually titrated back to a maintenance level of 1.5%. All animals received bilateral SLD injections of 400 μ L of adeno-associated virus (AAV) serotype 2/2 containing channelrhodopsin (AAV-CAG-ChR2-GFP) under control of the ubiquitous CMV early enhancer/chicken beta actin (CAG) promoter (UNC Vector Core, Chapel Hill, North Carolina). Stereotactic coordinates were measured from bregma and were based on a 15° head-down tilt and 10° lateral approach toward midline to avoid the transverse sinus: AP -11.0, DV -6.7, ML +2.2. During the same surgery, rats received bilateral implants of optical fibers (50- μ m core, numerical aperture 0.37, Thor Labs), with the fiber tip terminating at one end above the SLD, and the exposed end of the fiber terminating in a metal 2-mm-diameter ferrule (Precision Fiber Products, Inc., Milpitas, California) that was cemented in place. This allowed a light source to be attached at the time of experiment. Four EEG screws were also inserted into the anterior skull of each animal (Fig. 1A). These were inserted in a rectangular pattern 2-mm lateral from midline and 2-mm anterior, and 3-mm posterior, from bregma. Two electromyography (EMG) electrodes were inserted under the skin of the neck and above the nuchal muscles. EEG and EMG electrode terminals were inserted into a recording adapter and cemented in place using black acrylic cement (Fig. 1B).

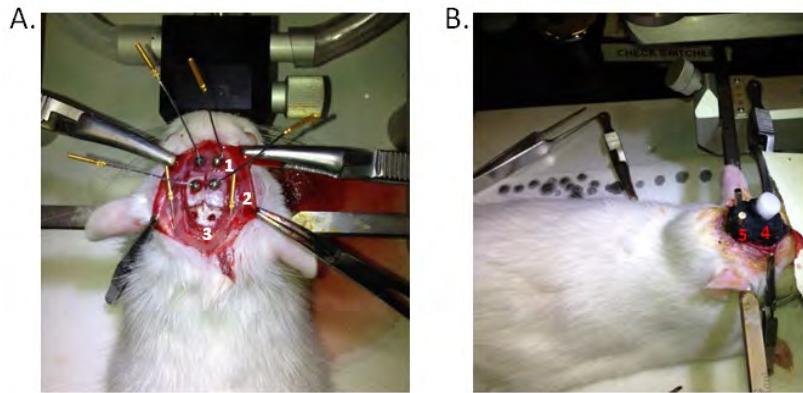


Fig. 1 Surgical procedure, as shown while the rat is anesthetized and head-restrained in the stereotaxic frame

Figure 1A:

- 1) Four EEG screws were inserted into the anterior skull to record brain activity.
- 2) Two EMG electrodes were embedded under the skin and above the neck muscles.
- 3) Two holes were drilled above the SLD and 400 nL of AAV-channelrhodopsin (ChR2) was injected bilaterally.

Figure 1B:

- 4) EEG and EMG electrode terminals were inserted into a physiological recording adapter and cemented into using black acrylic place.
- 5) 50-μm optical fibers were inserted through the same holes as viral injection (see item above 3) and terminated above the SLD. Fiber couplers were cemented in place with black acrylic.

Experimental design: After approximately 25 days of recovery the animals were placed in an operant chamber and allowed to freely move around the enclosure. Just prior to the start of each experimental trial, 2 fiber optic cables were mated to the 2 exposed ferrules using zirconia sleeves (Precision Fiber Products, Inc.) and the EEG and EMG leads were connected. These were then attached to an electro/optical commutator that allowed free rotation of the rats while simultaneously recording EEG and EMG signals and delivering optical stimulation.

Computer-controlled optogenetic lasers delivered 40-Hz pulses of blue light (447 nm) following 3 Protocols (Fig. 2A–C). Protocol No. 1 consisted of 16 laser

cycles of 1 min off/1 min on. Protocol No.2 consisted of 3.5 laser cycles of 5 min off/5 min on. Animals that showed no obvious change in locomotion during laser-on periods were excluded from further study. Non-excluded rats were run through both Protocols, beginning with No. 1.

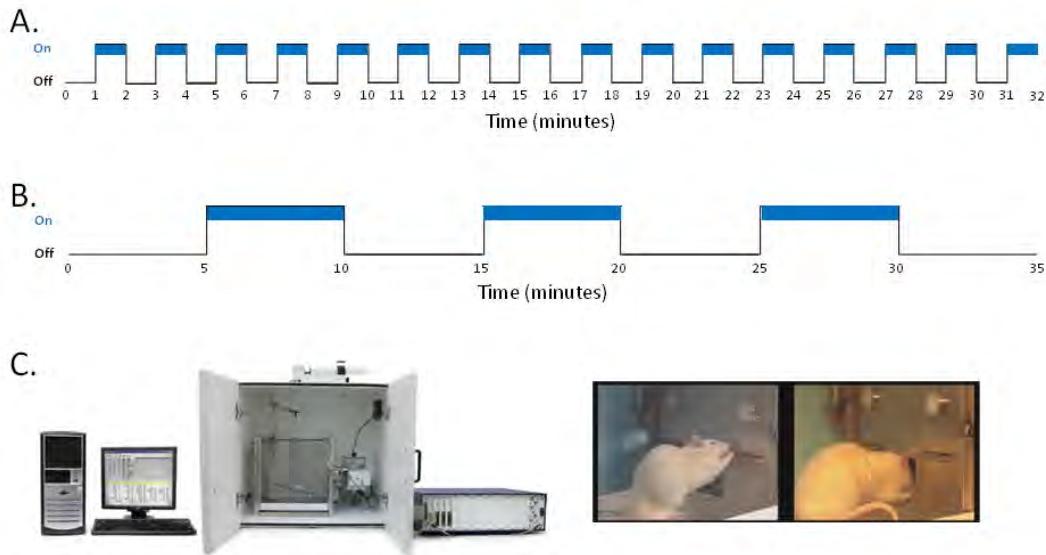


Fig. 2 Optogenetic stimulation paradigms

Blue lines and steps denote laser-on periods.

Figure 2A:

Protocol No. 1: Laser (40 Hz) 1 min off/1 min on (16 cycles). This paradigm was chosen to test the kinetics of SLD activation (i.e., rapid onset and offset), as well as the reliability and reproducibility of the effect.

Figure 2B:

Protocol No. 2: Laser (40 Hz) 5 min off/5 min on (3.5 cycles). This was chosen to test the impact of prolonged activation of the SLD and whether the effect could be sustained.

Figure 2C:

Protocol No. 3: Lever press for food, a test of motivation. Animals were trained to lever press for food inside of a conditioning box (left image). Levers are shown in the image on the right, as is the center food tray where the pellets are delivered. The blue-light laser was turned on for 1 min (6 trials) in an attempt to interrupt an ongoing trained, motivated behavior.

To test if activation of the SLD is sufficient to stop a motivated behavior, 3 rats were trained to lever-press for food pellets (Fig. 2C). These rats were food restricted to approximately 85% of their initial body weight by first reducing food intake to 1 pellet daily for 3 to 4 days, until the target weight was reached, after which additional food was given in order to maintain this weight for the remainder of the experiment. Rats were placed in a conditioning box and trained to press the left lever *ad libitum* for food pellet delivery (45 mg, BioServ). These training exercises consisted of 1 to 2 sessions per day, with 100 trials per session. Rats were judged to have reached criterion when they achieved 90% accuracy in any 20-trial block (i.e., no longer pressed the right control lever), a criterion met (and usually exceeded) by all rats in 4–8 sessions (i.e., 400–800 trials). Individual lever-presses were time-stamped and saved to a desktop computer for offline analysis. During the experimental phase, animals were connected to the laser and EEG and EMG recording adapter. Similar to the 2 Protocols previously described, optogenetic activation of the SLD was achieved by delivering 40-Hz pulses of blue light (447 nm) for 1 min with a 3-min inter-trial interval. Lever-press counts were made before, during and after laser stimulation to gauge the ability of the laser excitation to stop a trained, motivated behavior. All results are presented as mean plus or minus standard error.

Histology: Subsequent histological analysis was used to verify AAV injection location and fiber optic placement. Briefly, the rats were transcardially perfused with 10% formalin (100 mL over 5 min), followed by 10% formalin with 15% sucrose, for cryoprotection. Brains were sectioned at 40 μm and tissue stained for Nissl and green fluorescent protein (GFP) as described previously.¹⁷

Signal processing: Analysis of electrophysiological data (Offline Sorter, Neuroexplorer), and general data analysis (MATLAB) were performed using custom scripts.

3. Results

Despite over 50 years of REM sleep research, this report is the first to demonstrate that specific activation of SLD neurons is sufficient to rapidly induce REM atonia. For this purpose, we injected into the SLD an AAV expressing ChR2, thus making neurons in this vicinity excitable by blue laser light (447 nm) delivered through an optical fiber also aimed at the SLD. Use of ChR2 allowed us to selectively activate the neurons of interest without the confound of stimulating fibers of passage (e.g., electrical stimulation) or nonspecific neural activation (e.g., electrical stimulation and pharmaceuticals).

3.1 Rapid Induction of Muscle Atonia Following SLD Activation

Of the 6 animals that initially underwent surgery, 5 made it to the experimental stage, while the sixth rat dislodged the implanted optical fibers and was euthanized. In the remaining 5 rats, 3 (60%) showed varying degrees of behavioral effects following SLD stimulation at 20-mW peak laser strength, with 1 animal (CG4) showing pronounced atonia immediately following laser-onset (<1 s) at light intensities as low as 6 mW (Fig. 3). The 2 rats that showed no effect had misplaced AAV-ChR2 injections and/or fiber optic implants (as confirmed via histology), which prevented us from inducing atonia in these animals.

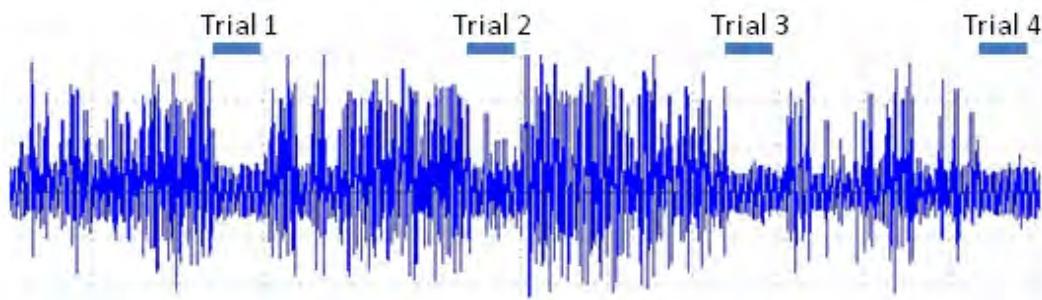


Fig. 3 EMG activity is dramatically reduced during laser-on trial periods, indicative of muscle atonia

Interestingly, this rapid decrease in muscle activity was not accompanied by changes in EEG frequency or amplitude, as illustrated in Fig. 4A and 4B. Possible explanations for this discrepancy are included in the following discussion.

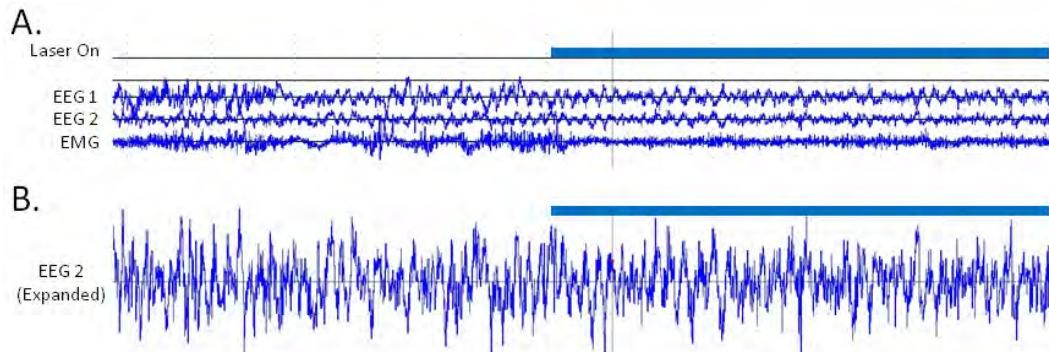


Fig. 4 EEG activity is not impacted during laser-on trial periods (rat CG4)

Figure 4A:

EEG and EMG activity are shown both before and during laser-on periods. While there is a rapid reduction in EMG amplitude, neither EEG channels show changes in activity.

Figure 4B:

Expanded view of EEG channel No. 2 to highlight continuity of signal.

3.2 Cessation of an Ongoing Motivated Behavior

Following our successful demonstration of a rapid inhibition in peripheral muscle activity, we next asked whether this muscle atonia was sufficient to immediately stop a motivated, trained behavior. For these experiments we trained the 3 responding rats to press the left lever in a conditioned chamber to receive food pellets *ad libitum* (see Section 2). Since the animals were initially food restricted to reduce body weight, the animals were highly motivated to perform this activity to receive additional volumes of food. Once trained, the animals pressed the lever at a near-ceiling rate of 8.5 ± 0.48 presses/15 s. This equates to an average interval of 1.76 s in between presses and encompasses the time it takes the animal to move to the food tray, quickly eat the food pellet and return to the lever to press again for another pellet delivery. At regularly spaced intervals, the laser was turned on using the same 40-Hz stimulation paradigm (Fig. 5A).

Optogenetic activation of the SLD resulted in an immediate cessation of lever-pressing in 2 out of 3 rats (CG3 and CG4), with the other rat (CG1) showing a reduction (but not complete cessation) in pressing during the laser-on condition. Results from CG3 are illustrated in Fig. 5A, with vertical green tick marks representing individual lever-presses. Note the rapid cessation of lever-pressing following the rapid induction of muscle atonia. The animal was able to continue pressing toward the end of the trials, albeit at a much reduced rate. This likely represents incomplete activation of the SLD, which will be discussed in detail in the following. Results from our exemplar rat (CG4) are summarized in Fig. 5B. Average lever press counts from 6 trials are shown in 15-s bins for the 2-min baseline period, 1-min laser-on condition (as denoted by the blue line) and the 1-min recovery period after stimulation was stopped. From this figure, it is clear that activation of the SLD is sufficient to immediately, and completely, cease an ongoing motivated behavior. Interestingly, following the termination of the laser, it took over 1 min for the animal to return to baseline lever pressing rates. This is suggestive of a lingering inhibition that reduces the animals' activity even in the absence of direct stimulation. The average lever pressing rate from the 3 animals, averaged over 6 trials each, is shown in Fig. 5C. The 60-s laser-on condition resulted in a significant reduction in lever press counts (2-way ANOVA, $p < 0.0001$), as compared to both the 60 s time periods before and after stimulation (baseline = 34 ± 1.94 ; laser = 10.67 ± 2.76 ; recovery = 30 ± 1.87).

Results from these experiments demonstrate the robust impact we can have over an animals' behavior by optogenetically activating the SLD to induce a REM atonia-like state. Not only were we able to stop the rat's natural exploratory behavior in a freely moving environment, but we were also able to immediately stop an ongoing motivated behavior using this approach.

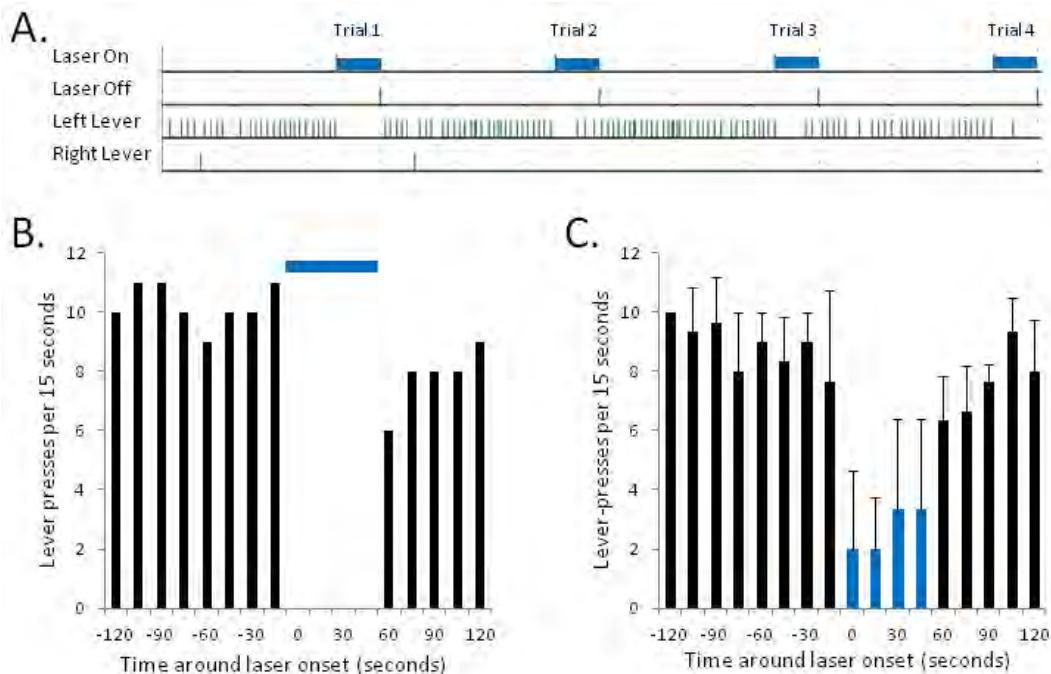


Fig. 5 Motivational lever pressing for food pellets is interrupted by activation of the SLD

Figure 5A:

Individual lever presses from rat CG3 are shown for 4 consecutive trials. The time of each 40-Hz stimulation trial is denoted by a blue bar. Note that the animal only made 2 "mistakes" by pressing the right lever, which does not result in food pellet delivery.

Figure 5B:

Average lever presses (rat CG4, 6 trials) are shown in consecutive 15-s bins for 2 min of baseline recording, 1-min stimulation and 1.25-min post-stimulation recovery. Activating the SLD completely abolished lever pressing in this animal.

Figure 5C:

Mean and standard deviation of lever presses from the 3 rats that responded to SLD activation by a decrease in muscle activity. Data are binned the same as in Fig. 5B.

3.3 Histological Identification of AAV Injections and Fiber-Optic Placement

Following the completion of the behavioral experiments, all animals were euthanized in order to perform histological analysis of the AAV injection locations and fiber-optic implantations. This information was then used to correlate the behavioral effects described above with anatomy. The anatomical location and spread of the AAV-ChR2 injection from our exemplar experimental rat (CG4) is illustrated by the black staining in Fig. 6A. The approximate location of the SLD is shown using red ovals (Fig. 6A and 6B). Based on this illustration, it appears that the left injection was dorsal to the target and likely outside the boundary of the SLD, while the fiber optic terminated too ventral. This is in contrast to the right injection that perfectly encompassed the SLD and surrounding regions. In rat CG3, the left injection was consistent with the boundary of the SLD, while the right side was located more ventral (Fig. 6B). This internal heterogeneity illustrates the difficulty of reliably hitting such a small brainstem structure without the advantage of genetically driven biomarkers. While the use of ChR2 allowed us to selectively activate the neurons of interest without the confound of stimulating fibers of passage or nonspecific neural activation (e.g., electrical stimulation and pharmaceuticals), we still need a better way to isolate these cells from surrounding structures. Thus, future experiments will look for biomarkers of these cells that could be used to drive AAV-ChR2 expression.

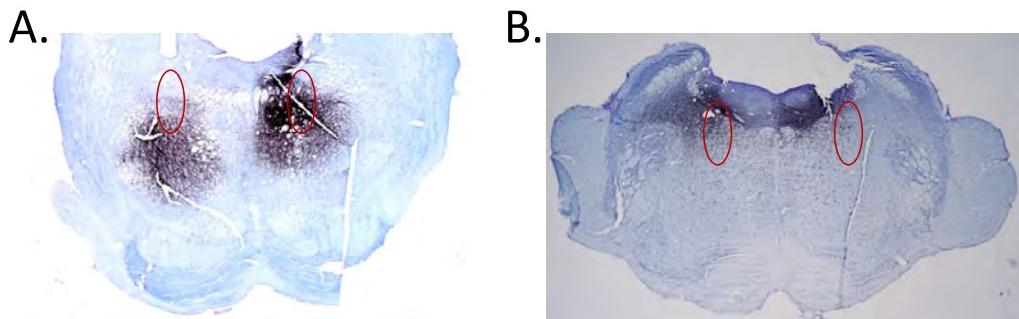


Fig. 6 Histological verification of AAV injection location

Figure 6A:

A coronal brain section from rat CG4: The blue Nissl stain highlights neuronal cell bodies, while the darker black is an anti-GFP stain to determine viral spread following AAV injection. Red ovals denote the approximate boundary of the SLD.

Figure 6B:

The coronal brain section from rat CG3: The blue Nissl stain highlights neuronal cell bodies, while the darker black is an anti-GFP stain to determine viral spread following AAV injection. Red ovals denote the approximate boundary of the SLD.

4. Discussion

The preliminary results presented here are the first to demonstrate such rapid and repeatable loss of muscle tone following optogenetic stimulation of the SLD. These data underscore the powerful influence that this nucleus has over postural muscle tone, both during waking and REM sleep. However, because there is no known way of targeting the ChR2 only to SLD neurons, we cannot know the exact types of neurons affected, and indeed it is highly likely that other neural types were influenced by our manipulation as well. To overcome this shortcoming, we would need to express ChR2 under control of gene promoters that are expressed selectively in the SLD.

One likely possibility for the inconsistent success rate (3 out of 5 rats) is the 5%–10% inherent variability in animal skull/brain size. Since our injections and fiber placements were 11-mm posterior from bregma, a 5% difference in brain size would produce an offset of 550 μm . The SLD is a small nucleus, only measuring approximately 600- μm anterior to posterior, so even perfectly consistent stereotaxic injections would produce unavoidable variation in both the efficacy of SLD targeting, as well as the degree to which SLD-targeted manipulations would instead reach other unrelated brain circuits. To better isolate our injections to the SLD neurons of interest and minimize activating surrounding structures, future studies will aim to identify functional genetic targets (e.g., receptors, transporters, etc.) that we can use to selectively modulate SLD neuron activity for both electrophysiological and optogenetic behavioral tests.

While EMG activity rapidly decreased following optogenetic activation of the SLD, there was no discernible change in EEG neural activity. Since the SLD is hypothesized to play a role in both the cognitive and peripheral components of REM sleep, these results initially appear to be in conflict. However, there are many explanations that could account for these differences (discussed in the following).

- 1) The SLD can be segregated into 2 main regions, with the anterior cells believed to project to the thalamus and activate the EEG while posterior cells project to deeper brainstem structures involved in muscle atonia. If our injections and fiber optics were solely located in the posterior SLD then it is possible that we dissociated the 2 components of REM.

- 2) For our experiments we activated the SLD while the animals were in an awake state, while REM sleep is normally entered following slow-wave sleep (SWS). In order to see changes in the sleep/wake component we may need to alter our experimental paradigm and test whether we can influence EEG activity after the animal is already in SWS.
- 3) EEG activity during waking and REM are nearly indistinguishable, thus it may be possible that we did drive the animal into a REM state but were unable to verify with the EEG.
- 4) Since the AAV-ChR2 expression was not driven by specific promoters of the SLD, it could be possible that we simultaneously activated the atonia circuitry and nearby competing wake nuclei, thus preventing the animal from entering REM sleep. Regardless of the reason, the lack of change in EEG activity does not distract from the profound impact we had on muscle tone.

Collectively, these preliminary experiments supplied us with behavioral and electrophysiological evidence as to the efficacy of activating the SLD to rapidly induce an atonic REM-like state.

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List of Symbols, Abbreviations, and Acronyms

AAV	adeno-associated virus
ChR2	channelrhodopsin
EEG	electroencephalography
EMG	electromyography
GFP	green fluorescent protein
Gi	gigantocellular nucleus
MS	medial septum
MUSC	Medical University of South Carolina
NIH	National Institutes of Health
NREM/REM	non-rapid eye movement to rapid eye movement
peri-LC α	peri-locus coeruleus α
PnC	pontis caudalis
PnO	pontis oralis
REM	rapid eye movement
SLD	sublaterodorsal
SubCD	dorsal subcoeruleus
SWS	slow-wave sleep
vSLD	ventrally located SLD

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